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Expression of Regeneration and Tolerance Factor on B Cell Chronic Lymphocytic Leukemias: A Possible Mechanism for Escaping Immune Surveillance

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Regeneration and tolerance factor (RTF) is a protein expressed on developing tissue such as the thymus and the placenta. RTF has been reported to down-regulate cell-mediated immune responses. To examine the potential role of tumor-derived RTF to suppressing antitumor responses, we analyzed a panel of seven B cell tumor lines for the membrane RTF using a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody, which reacts with membrane RTF. All the B cell tumor lines we examined express RTF on the cell surface. We also tested conditioned media from these B cell lines for their ability to suppress IL-2R expression on activated cells. Conditioned media from each B cell line suppressed IL-2R expression on activated Jurkat T cells and activated peripheral blood mononuclear cells. A monoclonal antibody to the biologically active portion of RTF reversed this suppressive activity. Finally, the tumor cell population from patients with chronic lymphocytic leukemia was found to express cell surface RTF. Thus, RTF expression could be a new mechanism used by tumor cells to escape immune surveillance. Am. J. Hematol. 61:46–52, 1999. © 1999 Wiley-Liss, Inc.

Key words: RTF (regeneration and tolerance factor); B cell lines; B-CLL

INTRODUCTION

One goal of administering antitumor vaccines and cancer immunotherapy is to enhance an existing cytotoxic immune response and destroy tumors. Antitumor responses have been enhanced by vaccines and IL-2 therapy with variable success rates. However, tumors can circumvent the autochthonous immune response and enhanced immune response in a variety of ways. One of the many mechanisms used is to redirect the cytotoxic immune response to a humoral response. This mechanism is used by a number of parasites [1] and most notably, by the placenta during pregnancy [2].

One mechanism used by the placenta for protecting a fetal allograft is by expression of regeneration and tolerance factor (RTF) [3]. RTF is a novel immunosuppressive molecule necessary for maintaining a viable pregnancy [4]. The placenta and fetal thymus express the highest amount of RTF [5]. Rubesa et al. [5] found that RTF is expressed in human first trimester decidual lymphocytes. RTF has distinct patterns of expression on lymphocytes during pregnancy. B cells from pregnant but not nonpregnant women express RTF [6]. However, lymphocyte expression of RTF is not always conducive for maintaining a viable pregnancy. When peripheral blood natural killer (NK) cells from women with a history of recurrent spontaneous abortion express RTF, this expression is predictive of an impending miscarriage [7]. How RTF-expressing NK cells are involved in altering the immunosuppressive environment within the placenta remains to be determined.

RTF has immunosuppressive properties in vivo. Extensive studies in murine models have also shown that RTF is necessary for immunosuppression or tolerance to a fetal allograft. Increased levels of RTF are present in

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uteri and lymph nodes draining uteri of pregnant mice compared with nonpregnant mice [8]. Moreover, treating pregnant mice with monoclonal antibodies to RTF results in fetal loss [3]. In vitro, RTF decreases proliferation of peripheral blood mononuclear cells (PBMC) in a mixed lymphocyte culture [9].

The immunosuppressive properties of RTF in maintaining fetal allografts may also be a factor for tumor survival in vivo. We also have shown that the B cell tumor line CCRF-SB (SB) expresses RTF mRNA as well as protein [6]. Because the B cell tumor line SB expressed RTF mRNA and protein, and B cells from pregnant women express RTF, we studied RTF expression on a panel of B cell lines. We determined the immunosuppressive effects of conditioned media from the B cell lines expressing RTF on IL-2R expression in activated Jurkat T cells and activated PBMC. We also tested for the presence of soluble RTF in the B cell line conditioned medium by ELISA assay. Finally, we examined the tumor cell populations from eight patients with B cell chronic lymphocytic leukemia (CLL) for RTF expression.

MATERIALS AND METHODS

Cell Lines

Cells lines were maintained in RPMI Medium 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies), 100 units/ml penicillin G (Life Technologies), 100 μg/ml streptomycin sulfate (Life Technologies), 10 μM 2-mercaptoethanol and L-glutamine (Sigma Chemical Company, St. Louis, MO). Cells lines were grown in a 5% CO_2 atmosphere at 37°C. PLL-1, PLL-2, and CLL-1 were gifts from S. Finerty and A.B. Rickinson (University of Bristol, Bristol, England). E55-7G was a gift from Earl E. Henderson (Temple University School of Medicine, Philadelphia, PA). SB, Daudi, and RPMI-8866 were purchased from the American Type Culture Collection (Rockville, MD).

Patient Population

The study included eight patients diagnosed with B cell CLL (B-CLL). Informed consent was obtained from patients. These patients' characteristics were described previously by Aller et al. [10]. Peripheral whole blood samples from these patients were collected in sodium heparin. Lymphocytes were isolated by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density-gradient centrifugation and frozen until analysis [10].

Monoclonal and Polyclonal Antibody Generation

Monoclonal antibodies were generated to the membrane form and the soluble form of RTF as described previously [11]. The monoclonal antibody 2C1 was generated using a synthetic peptide (Clonotech, Palo Alto, CA), which represents amino acids 488-514 of RTF. The monoclonal antibody F3C was generated using a synthetic peptide (Clonotech), which represents amino acids 110-120 of soluble RTF. Polyclonal antiserum to soluble RTF was generated in rabbits using the synthetic peptide (Clonotech), which represents amino acids 110–120 of soluble RTF as described previously [11]. The specificity of each antibody was confirmed by ELISA and confirmed by the demonstration that preincubation of the antibody with the peptide abolished membrane binding in tumor lines.

Immunoglobulin G (IgG) was purified from ascites or rabbit serum using a High Trap-G column (Pharmacia Biotech, Inc., Uppsala, Sweden) according to the manufacturer's directions. Purified IgG from 2C1 was conjugated to fluorescein isothiocyanate (FITC) (Sigma) as described previously [12].

RTF Expression

B cell tumor lines were stained with the 2C1-FITC for 20 min on ice, fixed with fixative according to manufacturer's directions (Coulter Corp., Miami, FL). Five thousand cells were collected and analyzed on the Coulter EPICS XL-MCL (Coulter). PBMC from patients diagnosed with B-CLL were labeled for RTF using the monoclonal antibody 2C1-FITC, and either an R-phycoerythrin (RPE)-conjugated monoclonal antibody to kappa (Dako Corp., Carpinteria, CA) or lambda light chain (Dako). Samples were also labeled for expression of CD19/CD5 (FITC/PE) (Ortho Diagnostic Systems, Raritan, NJ) and CD3 (FITC) (Coulter). Before data acquisition, 1 μg/ml propidium iodide was added for detecting dead cells. Five thousand live cells were collected and analyzed on the Coulter EPICS XL-MCL.

Conditioned Media

Three hundred thousand cells from each tumor cell line were incubated in six well plates in RPMI-10% FCS for 4 days at 37°C. Culture supernatants were harvested and cells removed by centrifugation at 1,000 *g* for 10 min. Supernatants were stored at −20°C.

Conditioned Media Co-Cultures of PBMC and Jurkat Cells

A 96 well microtiter plate was coated with 20 μl of 1:200 dilution of anti-CD3 (Coulter) overnight at 4°C and washed three times with PBS before adding PBMC or Jurkat cells. PBMC were isolated from peripheral blood using Ficoll-Paque and Jurkat cells were harvested from tissue culture flasks. Cells were seeded at 1×10^5/well in 100 μl. For Jurkat cells, 10 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) was also added to each well. Fifty microliters of conditioned medium from each B cell line expressing RTF or 50 μl RPMI was

added to each culture. Either 10 μl of F3C ascites or normal mouse IgG_1 (PharMingen, San Diego, CA) was added to appropriate wells.

After 24 hr of incubation, PBMC and Jurkat cells were harvested and washed twice with RPMI-10FCS. Cells were stained for IL-2 receptor expression using the monoclonal antibody, IL-2R1-RD1 (Coulter). After staining for 20 min at room temperature, the cells were washed and fixed with fixative (Coulter). Data from 5,000 events were acquired and analyzed on an Ortho Cytoron Absolute (Ortho Diagnostics Systems).

Soluble RTF ELISA Assay

A 96 well Immulon-2 plate (Dynatech Laboratories, Inc., Chantilly, VA) was coated with 400 ng/well of a polyclonal, rabbit antisoluble RTF antibody overnight at 4°C. After blocking for 1 hr at room temperature with 0.1% bovine serum albumin, 50 μl of five-fold serial dilutions of each conditioned medium were incubated 1 hr at room temperature. After washing, the monoclonal antibody, F3C, was added for 1 hr at room temperature followed by goat antimouse IgG-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). Diethanolamine substrate, pH 9.8, was added. Optical density was measured at 450 nm after 30 min.

Statistical Analysis

RTF expression by flow cytometry was expressed as the mean channel index (MCI). The MCI was calculated as a ratio by dividing the mean log channel fluorescence of the 2C1-FITC stained cell lines by the mean log channel fluorescence of FITC-conjugated-isotype matched control.

CD25 expression on activated cells incubated with conditioned medium was expressed as a percentage of maximal response of activated cells without conditioned media. Percent maximal response compared with activated cells was calculated as follows: 100 × (%CD25 expression of activated cells in the presence of conditioned medium)/(%CD25 expression of activated cells in presence of RPMI-10% FCS).

Chi-squared analysis was used to evaluate the four experiments with the effects of conditioned media compared with activated cells without conditioned media. A P value less than 0.05 was considered statistically significant.

Soluble RTF titers by ELISA were expressed as the reciprocal titer that had an optical density greater than background. Background optical density was measured in wells in which conditioned medium was omitted.

RESULTS

Expression of RTF on Lymphocytic Tumor Cell Lines

Because of the previous findings that RTF was expressed on the leukemic cell line SB [6], we evaluated RTF expression on a panel of B cell tumors. As shown in Figure 1, all of the B cell lines expressed high levels of RTF. RTF was expressed at different densities on each cell line we examined as indicated by MCI (Table I). In each case the MCI was unique to the tumor cell population studied as shown in Table I. In addition, the relative expression of RTF correlated with the stage of B cell development. The least differentiated B cell line, PLL-1, expressed the most RTF (MCI = 133.9). The more differentiated cell lines, RPMI8866 and CLL-1, had MCI of 19.6 and 10.9, respectively. As described previously, B cells from normal peripheral blood do not express membrane RTF. [13]

The Effects of B Cell Tumor Conditioned Media on CD25 (IL-2R) Expression on Activated Jurkat T Cells and PBMC

The biological activity of RTF resides in the smaller, soluble peptide. We have shown previously that RTF decreased thymidine incorporation in stimulated lymphocytes [11]. Because B cell tumor lines express surface RTF, we tested the conditioned medium from each B cell tumor line to determine if secreted, soluble RTF could down-regulate an in vitro immune response. In these studies, we first examined the effects of B cell conditioned medium on lymphocyte activation. The Jurkat T cell line or PBMC were each activated in the presence and absence of B cell conditioned medium for 24 hr. Cells were harvested, stained for CD25 expression as an activation marker and analyzed by flow cytometry. Supernatants of conditioned medium of each B cell line tested down-regulated CD25 expression on Jurkat T cells and PMBC (Fig. 2).

Tumor lines produce any number of immunosuppressive molecules. Thus, we determined whether antibody to soluble RTF could reverse this immunosuppressive effect. For each experimental sample studied, the suppressive effect was reversed by addition of the monoclonal antibody to RTF, F3C, to cultures ($P < 0.001$) (Fig. 2). Therefore, the immunosuppressive activity was specific because of RTF.

We next confirmed that each supernatant contained RTF and semiquantitated RTF protein. Each B cell conditioned media contained the biologically active, soluble portion of RTF by ELISA assay (Table II). Titers ranged from 125 for CLL-1, E55-7G, PLL-1, RPMI-8866 and SB to 625 for Daudi, PLL-2 (Table II).

Expression of RTF on Tumor Cells From Patients With CLL

Because B cell tumor lines express RTF, we investigated whether the tumor population from patients with CLL expressed RTF. We used monoclonal expression of either the kappa or lambda light chain as our marker for tumor clonality. The defined tumor population from pa-

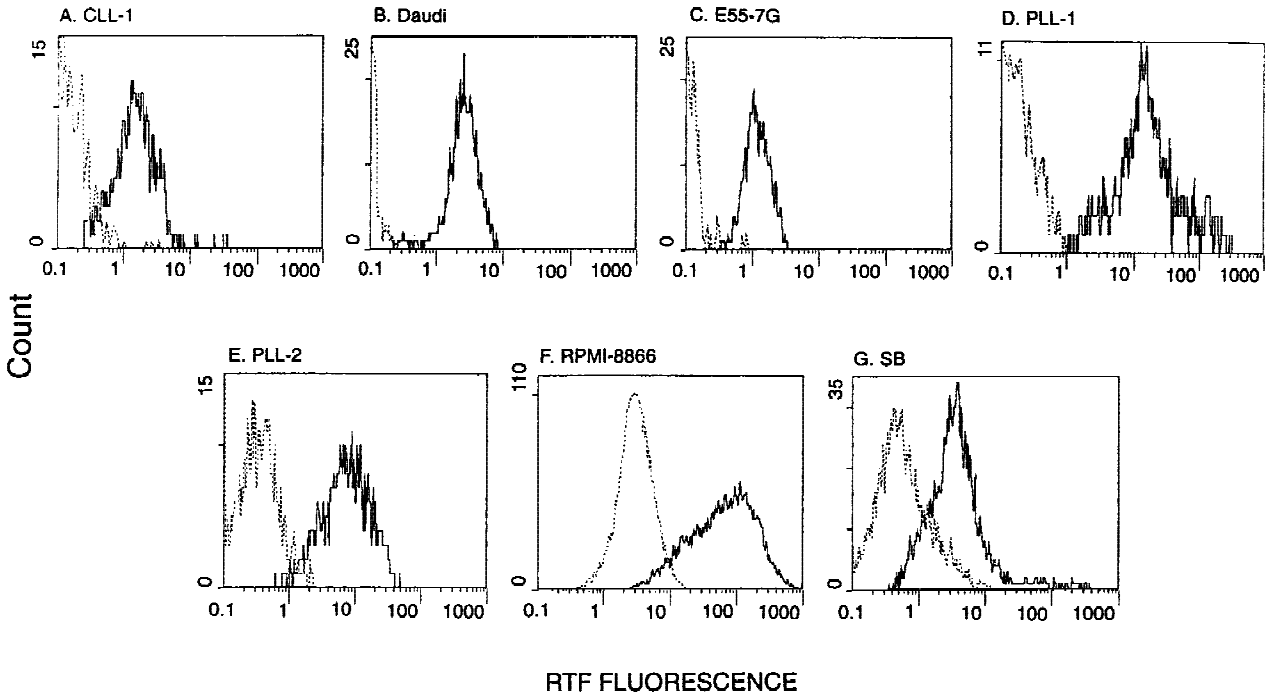

Fig. 1. Expression of RTF on B cell tumor lines. The anti-RTF monoclonal antibody, 2C1, was conjugated with FITC and incubated with B cell tumors indicated by the solid line (——). The isotype control is indicated by the dashed line (----). The cells were analyzed on the Coulter Epics XL-MCL flow cytometer. Relative cell surface expression of RTF is expressed as the MCI. Cell counts are shown on the Y-axis and RTF fluorescence on a log scale is shown on the X-axis.

TABLE I. RTF Expression on B Cell Tumor Lines*

Cell line	Stage	RTF MCI[a]
CLL-1	Plasmacytoid B cell	10.9
Daudi	Intermediate B cell	18.1
E55-7G	Intermediate B cell	11.1
PLL-1	Pre-B cell	133.9
PLL-2	Early B cell	22.8
RPMI-8866	Mature B cell	19.6
SB	Intermediate B cell	5.4

*RTF, regeneration and tolerance factor; MCI, mean channel index; CLL, chronic lymphocytic leukemia.
[a]MCI = Ratio of sample mean log channel:control mean log channel.

tients with CLL expressed membrane RTF in each of the samples studied. Coexpression of membrane RTF and either the kappa or lambda light chain (Table III) identified the leukemic cells. RTF expression on leukemic cells ranged from 20.5% to 93.2% and was equivalent to the percentage of CD19/CD5+ B cells (r = 0.9). In contrast and as shown in previous studies [7,13] less than 4% of normal PBMC expressed RTF. Dual parameter dot plots for patients no. 2 and no. 7 from Table III are displayed graphically in Figure 3. As shown in Figure 3, the tumor population from patient no. 2 coexpressed RTF and the lambda light chain. The tumor population from patient no. 7 coexpressed RTF and the kappa light chain. These leukemias also coexpressed the B cell marker CD19 and the T cell marker CD5, which are diagnostic for CLL (Table III).

DISCUSSION

These studies have shed light on a novel immunosuppressive cytokine, RTF. RTF is expressed on the surface of seven B cell leukemia lines representing various stages of differentiation as well as peripheral blood B lymphocytes from patients diagnosed with B cell CLL. Tissue culture supernatants from all these B cell lines suppressed IL-2R expression on Jurkat cells and PMBC activated with PMA. Antibody to RTF reversed this suppressive effect suggesting that RTF was secreted by these B cell lines and could suppress T cell activation.

We demonstrated RTF expression for a panel of B cell tumor lines using flow cytometry. A comparison of the RTF MCI indicated that RTF was present at different densities on the surface of these cell lines. RTF expression correlated with B cell lineage maturation. Early B cell lines express higher levels of RTF than intermediate or plasmacytoid B cell lines. The cell line that was the least differentiated, PLL-2, expressed RTF levels of an MCI = 133.9. An exception was the SB line, which expressed the least RTF (MCI = 5.4). Because normal, circulating PBMC do not express RTF, RTF expression

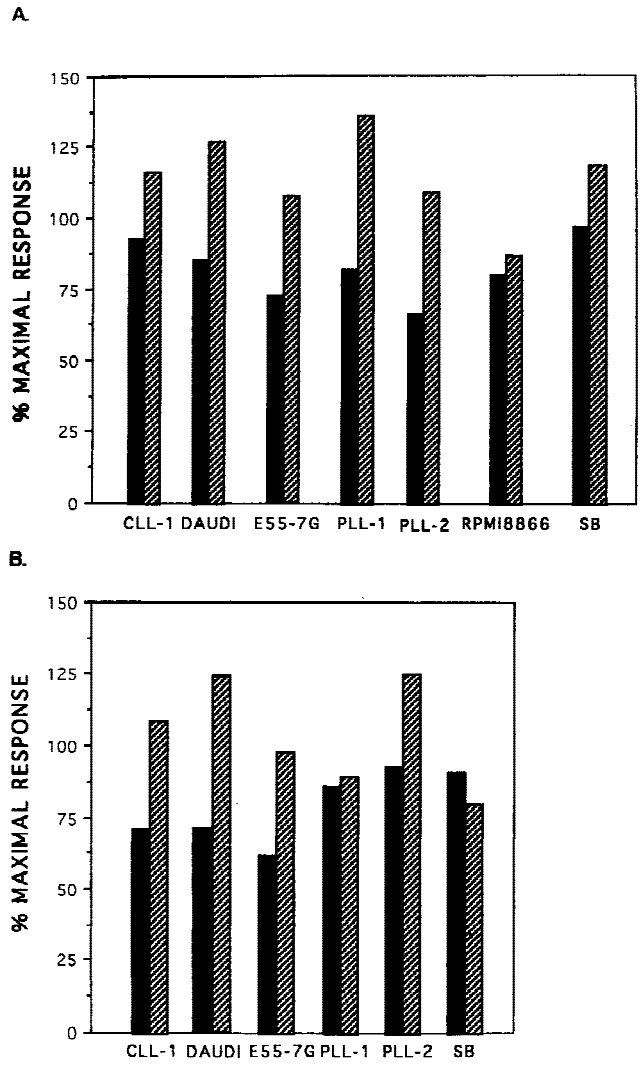

Fig. 2. The effects of B cell tumor conditioned media on CD25 expression of activated Jurkat T cells and PBMC. Conditioned media from each B cell tumor line were incubated with anti-CD3, PMA-activated Jurkat T cells (A) and PMA-activated PBMC (B). Twenty-four hours later, cell suspensions were examined for CD25 expression. The samples were analyzed on an Ortho Cytoron Absolute. The percent maximal response for each cell culture supernatant compared with activated cells without cell culture supernatants is represented on the Y-axis. Results for each culture supernatant are represented on the X-axis.

may be related to the stage of a particular B cell during hematopoiesis.

Tumor and host immune cell interactions play a critical role in tumor progression. It has been shown that tumors may produce immunosuppressive molecules such as cytokines that affect the balance of host interactions and shift the immune response towards tumor survival. A number of these molecules have been identified. For instance, ovarian carcinomas produce TGF-β and IL-10 [14]. Primary renal cell cultures supernatants produced

TABLE II. Soluble RTF Protein for B Cell Tumor Lines*

Cell line	Soluble RTF in conditioned media[a]
CLL-1	125
Daudi	625
E55-7G	125
PLL-1	125
PLL-2	625
RPMI-8866	125
SB	125

*See Table I.
[a]Titer = 1/dilution with OD_{450} greater than medium alone.

TABLE III. Expression of RTF on PBMC From Patients With CLL

	Percentage of lymphocytes expressing				
Patient	RTF MCI[a]	RTF/kappa	RTF/lambda	CD19/CD5	CD3
1	2.5	77.8	1.4	97.4	1.5
2	3.0	2.0	88.3	74.8	7.6
3	2.6	0.8	86.6	83.9	5.1
4	2.7	1.3	93.2	95.3	2.6
5	1.9	0.7	79.8	88.0	8.2
6	1.3	88.0	0.2	99.1	1.9
7	3.8	80.9	0.1	93.9	2.7
8	2.5	20.5	1.7	18.5	73.7

*See Table I.
[a]MCl = ratio of sample mean log channel:control mean log channel.

IL-6, IL-10, IL-11 and TGF-β [15]. In addition, the primary culture supernatants either enhanced or suppressed T cell blast transformation [15]. New immunosuppressive molecules produced by tumors have also been identified. For example, a 14kD immunosuppressive protein was derived from a human melanoma cell line. This protein inhibited PBMC proliferation and IL-2 secretion [16]. We have found that the novel immunosuppressive protein, RTF, is expressed on tumor lines and tumors from patients with CLL.

RTF is an immunosuppressive molecule described previously on the SB tumor cell line [6]. The biological properties of RTF reside in the smaller, soluble portion of RTF that is proteolytically cleaved at the N-terminus [11]. Previously, we characterized RTF activity using a recombinant product and found that RTF modulates NK activity and lymphokine production. Recombinant, soluble RTF down-regulates NK activity in a dose-dependent fashion at each effector to target ratio tested (unpublished observations, Sum et al.). The recombinant product suppresses CD25 expression on activated Jurkat T cells and also suppresses IL-2 but not IFN-γ production (unpublished observations, Lee and Beaman).

In this study, the B cell tumors secreted soluble RTF into tissue culture medium. We examined these preparations to determine if they might contain RTF and to

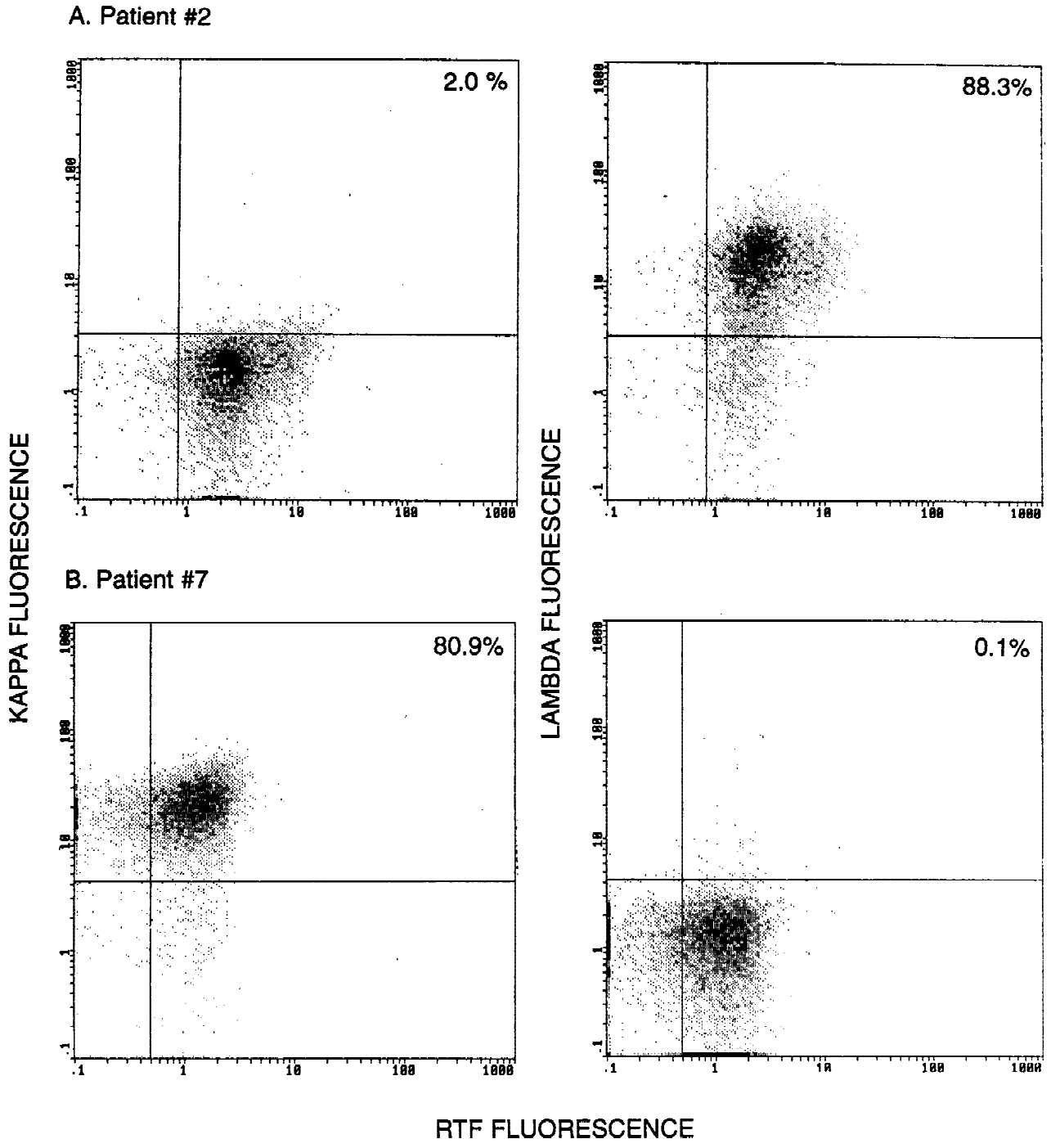

Fig. 3. RTF expression on PBMC from patients with CLL. Peripheral blood samples from patients diagnosed with CLL were stained for the expression RTF using 2C1-FITC in conjunction with either antibodies to the kappa light chain or lambda light chain. The samples were analyzed on a Coulter Epics XL-MCL. Kappa or lambda light chain expression is represented on the Y-axis. RTF fluorescence is represented on the X-axis.

determine if RTF could be responsible for the down-regulation of the immune response that is common in both the placenta and tumors. Supernatants of conditioned medium suppressed CD25 expression of activated Jurkat T cells and activated PBMC. Conversely, CD25 suppression was reversed using a monoclonal antibody to soluble RTF. These data suggest that RTF secreted by tumors may effect cellular activation by down-regulating CD25 expression and subsequently lymphokine secretion.

In these studies we show that RTF is expressed on B cell CLL. Leukemic cells from all eight clinical blood

samples expressed measurable levels of RTF. RTF expression may reflect the fact that these leukemias are arrested in B cell development. These data suggest to us that B cells may express RTF for protection from immune destruction in the bone marrow possibly by down-regulating or suppressing proliferation of lymphocytes that may kill the tumor. Alternatively, RTF may simply be a marker for developing B cells. Whether RTF is a mechanism for protection of tumors from the immune system or simply a developmental marker, this is the first description of a placental-associated protein in leukemias. Studies are planned to evaluate the mRNA expression as well as the expression of soluble RTF from freshly cultured lymphocytes. Finally, we plan studies to correlate RTF expression with patient outcome and the possible use of RTF as a prognostic indicator.

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